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18 Wheeler regulates apical constriction of salivary gland cells via the Rho-GTPase-signaling pathway

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Abstract

Rho GTPase and its upstream activator, guanine nucleotide exchange factor 2 (RhoGEF2), have emerged as key regulators of actin rearrangements during epithelial folding and invagination (Nikolaidou, K.K., Barrett, K. (2004). A Rho-GTPase-signaling pathway is used reiteratively in epithelial folding and potentially selects the outcome of Rho activation. *Curr. Biol*. 14, 1822–1826). Here, we show that *Drosophila* 18 wheeler (18W), a Toll-like receptor protein, is a novel component of the Rho-signaling pathway involved in epithelial morphogenesis. *18w* Mutant embryos have salivary gland invagination defects similar to embryos that lack components of the Rho pathway, and ubiquitous expression of 18W results in an upregulation of Rho signaling. Transheterozygous genetic interactions and double mutant analysis suggest that 18W affects the Rho-GTPase-signaling pathway not through Fog and RhoGEF2, but rather by inhibiting Rho GTPase activating proteins (RhoGAPs). We show that RhoGAP5A and RhoGAP88C/Crossveinless-c (CV-C) are required for proper salivary gland morphogenesis, implicating them as potential targets of 18W.

Keywords: 18 Wheeler; Rho GTPase; RhoGEF; RhoGAP; *Drosophila*; Invagination; Salivary gland; Tubulogenesis

Introduction

Perfectly orchestrated cytoskeletal reorganizations are crucial for morphogenetic movements and, consequently, the proper development of the entire *Drosophila* embryo. Cells that internalize during development, such as those that form the ventral furrow, posterior midgut, or salivary gland, require coordinated actin–myosin-based contractility at their apices. During morphogenesis, apical localization and contraction of actin–myosin is controlled by the Rho-GTPase-signaling pathway (Dawes-Hoang et al., 2005). The *folded gastrulation* (*fog*) gene is thought to encode the ligand for a signaling cascade that controls cell invaginations through this Rho pathway (Barrett et al., 1997; Costa et al., 1994). Concertina (CTA), an alpha G protein subunit of the Gá12/13 class, acts downstream of FOG and likely activates RhoGEF2, thereby promoting the exchange of GDP for GTP on Rho and favoring Rho activation. RhoGAPs work in opposition to RhoGEF2 by catalyzing Rho's intrinsic hydrolysis of bound GTP to GDP and limiting Rho activation (Sanny et al., 2006). Determining which of the 20 RhoGEFs and 21 RhoGAPs (Billuart et al., 2001) are involved in epithelial invagination will be central to understanding epithelial organ morphogenesis.

The *Drosophila* embryonic salivary gland serves as an ideal model for identifying genes involved in the process of epithelial invagination. Salivary glands begin as two single-layered epithelial discs, called salivary placodes, on the ventral surface of the embryo in parasegment 2 (Figs. 1A, B, arrowheads). Expression of the homeotic gene *Sex combs reduced* (*Scr*) in parasegment 2 is necessary for the specification of the salivary primordium; *Scr* mutant embryos lack salivary glands, while *Scr* misexpression in other parasegments can lead to ectopic salivary gland formation (Panzer et al., 1992). Following salivary gland specification, the salivary placodes invaginate via apical constriction at their dorsal posterior corners (Fig. 1C, arrowhead) and internalize dorsally at a 45° angle (Fig. 1D). Once internalized, the salivary glands change direction and migrate posteriorly (Fig. 1F) until they lie horizontally inside

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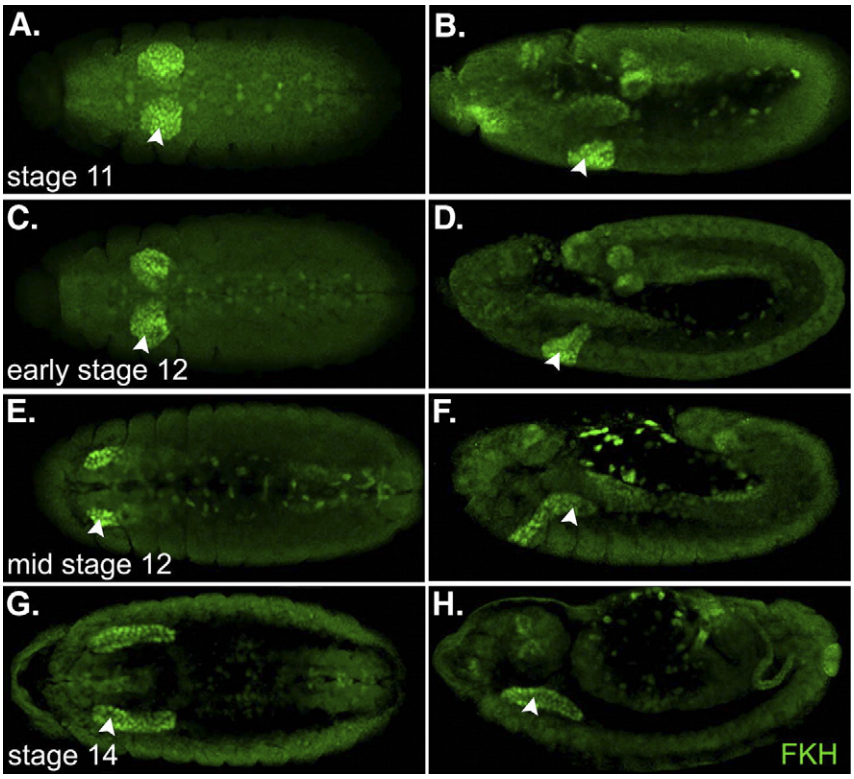

Fig. 1. Stages of salivary gland development. Salivary gland cells (arrowhead) are stained for FKH in green. (A, C, E, G) Ventral and (B, D, F, H) the corresponding lateral views of developing salivary glands from stages 11 through 14. (A, B) At stage 11, salivary gland cells are on the ventral surface of the embryo. (C, D) During early stage 12, salivary gland cells begin to invaginate dorsally towards the interior of the embryo at roughly a 45° angle. (E, F) Upon reaching the interior of the embryo, the salivary glands change direction and migrate posteriorly throughout stages 12 and 13. (G, H) By stage 14, the salivary glands have fully internalized and reached their final position within the embryo; parallel to one another and oriented in the anterior–posterior axis.

the embryo (Figs. 1G, H) (Bradley et al., 2003; Myat and Andrew, 2000b).

A great deal has yet to be discovered about the process of salivary gland invagination. Thus far, only two transcription factors, Fork head (FKH) and Huckebein (HKB), and two components of the Rho-mediated signaling pathway, FOG and RhoGEF2, have been shown to be required for salivary gland invagination (Lammel and Saumweber, 2000; Myat and Andrew, 2000a, 2002; Nikolaidou and Barrett, 2004). To identify additional genes involved in this process, we performed microarray experiments with *Scr* mutant embryos. Since SCR is the primary initiator of salivary gland specification (Panzer et al., 1992), genes downregulated in *Scr* mutants are potentially involved in many aspects of salivary gland development, including invagination.

One gene we identified in these experiments encodes a Toll-like receptor protein called 18 wheeler (18W) (Eldon et al., 1994). The large family of mammalian Toll-like receptor (TLRs) has been shown to function in anti-microbial resistance. In *Drosophila*, however, Toll is the only one of the nine *Drosophila* Toll family members (Toll, 18W, Toll-3 to -9) that is involved in immunity (Lemaitre et al., 1996). The role of the other eight Toll family members remains largely undetermined. Since most of these are expressed during embryogenesis in cells that migrate, change shape, or change neighbors (Eldon et al., 1994; Gerttula et al., 1988; Kambris et al., 2002), *Drosophila* Tolls are predicted to have important functions during embryonic development. In support of this hypothesis, Toll is important not only for immunity but also for embryonic dorsal–ventral patterning, muscle development, and proper motoneuron innervation (Anderson et al., 1985; Halfon et al., 1995; Hashimoto et al., 1988; Rose et al., 1997). Although 18W appears to be important for larval fat body development and follicle cell migration, no defects in tissues of *18w* mutant embryos have been identified, and no mechanisms for 18W action have been demonstrated (Kleve et al., 2006; Ligoxygakis et al., 2002; Williams et al., 1997).

Here we show that *18w* is required during embryonic salivary gland invagination. To our knowledge this is the first demonstration of an important role during embryonic development for any of the eight remaining, elusive members of the Toll family. We provide both genetic and biochemical evidence that 18W controls salivary gland invagination by acting as a positive regulator of the Rho-GTPase-signaling pathway. We also show that 18W is not a component of the FOG/RhoGEF2 pathway that activates Rho but may regulate Rho by inhibiting its inhibitors, the RhoGAPs. Additionally, we identify two RhoGAPs, RhoGAP5A and RhoGAP88C/Crossveinless-c (CV-C), that are involved in salivary gland invagination and, therefore, are potential targets of 18W regulation.

Materials and methods

Fly strains

The following mutants and transgenic stocks were used in this study: *18w*$^{\Delta 21}$ (provided by E. Eldon), *sqhE20E21* (Winter et al., 2001), *cv-c*^{M62} (Denholm et al., 2005), *fog*^{4a6}, *UAS-dsRNA RhoGAPs* (Billuart et al., 2001), *UAS-fog* (Dawes-Hoang et al., 2005), *UAS-18w* (provided by J. Reichhart), *scabrous-GAL4* (provided by M. Foss), *daughterless-GAL4* (provided by D. Bilder). *Scr*$^{\varepsilon K6}$, *rhoGEF2*^{04291} and *18w*$^{\Delta 7-35}$ were obtained from the Bloomington stock center. *w*^{1118} flies were used as wild-type controls for all experiments.

Immunocytochemistry and in situ hybridization

Embryo fixation and staining were performed as described (Chandrasekaran and Beckendorf, 2003). The salivary gland lumen-specific antibody used was mouse anti-CRUMBS (Cq4) (Developmental Studies Hybridoma Bank, University of Iowa) at 1:25. The salivary gland nuclear-specific antibody used was rabbit anti-FKH at 1:1000. Mouse anti â-galactosidase was also used at 1:1000 (Roche). Alexa Fluor-546 and -488 (Molecular Probes) secondary antibodies were used at 1:500 and visualized by the Zeiss 510 confocal microscope.

Whole-mount *in situ* hybridization was performed as described (Tautz and Pfeifle, 1989) with modifications (Harland, 1991) using antisense digoxigenin-labeled probes. BCIP and nitro blue tetrazolium were used as substrates for alkaline phosphatase to visualize the signal. After being rinsed, cleared with 50% glycerol and then 70% glycerol, embryos were visualized and photographed using Nomarski optics on the Leica DMRB microscope.

Biochemistry

The protein embryonic extracts were prepared by homogenizing thirty embryos per sample in 2× SDS gel loading buffer (100 mM Tris–Cl pH 6.8, 200 mM dithiothreitol, 4% SDS, 0.2% bromophenol blue, 20% glycerol). Proteins from whole extracts were separated by SDS-PAGE (15% acrylamide), blotted to PVDF membrane (Immun-Blot; Bio-Rad), and probed with affinity purified rabbit anti-phospho-Sqh (Matsumura et al., 1998) at 1:200 and mouse anti-HP1 at 1:2000. Membrane-bound antibodies were detected with Alexa fluor-680 and -800 (Molecular Probes) used at 1:40,000 and analyzed with an Odyssey infrared imaging system (Li-Cor Biosciences, Inc.).

Results

18w RNA is expressed in invaginating salivary gland cells and is dependent on SCR

Based on our microarray experiments, *18w* is downregulated in *Scr* mutants, implicating it in salivary gland development. To follow *18w* expression in more detail, we performed RNA *in situ* hybridizations. The results show that in wild-type embryos *18w* RNA is not maternally contributed but is expressed in salivary gland cells prior to and throughout their invagination. Within the salivary placode, *18w* expression first becomes evident at stage 11 as a small spot in the dorsal posterior region (Fig 2A). During early stage 12, following the beginning of invagination, *18w* expression spreads throughout the placode (Fig. 2B). During the remainder of stage 12, *18w* transcripts can be detected in both gland cells that have yet to invaginate as well as those that have already internalized, albeit at a reduced intensity (Figs. 2C–E). At stage 13, *18w* transcripts cease to be expressed in salivary gland cells but are evident in salivary duct cells (Fig. 2F). In addition to salivary glands and ducts, *18w* RNA is also detected in other tissues undergoing morphogenesis, including the tracheal placodes and the hindgut (Eldon et al., 1994). As anticipated from our microarray experiments, *18w* transcripts are absent exclusively from parasegment two in *Scr* mutants while expression in the rest of the embryo remains unaltered (Figs. 3B, D). Overall, performing microarray experiments with *Scr* mutant embryos has proven to be an effective method for identifying salivary gland genes.

18w mutant embryos exhibit salivary gland invagination defects

While *18w* is expressed in several tissues undergoing morphogenesis, embryonic defects have yet to be identified in *18w* mutant embryos. The striking *18w* expression within the invaginating salivary gland prompted us to investigate more carefully the role of *18w* in embryonic salivary gland development. In examining a null allele of *18w*, *18w*$^{\Delta 21}$, we discovered that initiation of invagination in the dorsal posterior region of the placode appears normal (data not shown). However, during the next phase of invagination, in which the remaining cells of the salivary placode normally internalize in a strict sequential order, defects become apparent. In *18w* mutants invagination is less synchronized; too many placode cells invaginate simultaneously rather than sequentially, thereby causing a wider lumen in 66% ($n=50$) of *18w* mutants when compared to wild-type embryos (compare Figs. 4A and B, Table 1). Thus, it is the timely progression of invagination, not the invagination process itself, that is affected in *18w* mutants. Due to the lack of proper coordination in *18w*, the internalization of the gland cells appears to be delayed (Figs. 4D, F) causing the cells that internalize last to remain near the ventral surface instead of reaching their final, more dorsal, position within the embryo. Eventually all of the salivary gland cells do internalize in *18w* mutants, but the proximal part of the gland, which remains abnormally close to the ventral surface, becomes caught in the anterior movement of the ectoderm during head involution. As a result, the glands end up much closer to the anterior end of the embryo than in wild-type embryos (Fig. 4H). Similar defects are seen in mutants homozygous for the loss of function *18w* allele, *18w*$^{\Delta 7-35}$, and in *18w*$^{\Delta 21}$, *18w*$^{\Delta 7-35}$ transheterozygous mutant embryos (data not shown). Based on these results, *18w* is an important component in coordinating salivary gland invagination.

18W is a novel component of the Rho-mediated signaling pathway

The Rho-mediated signaling pathway is required for proper invagination of the ventral furrow, posterior midgut cells, and salivary glands (Barrett et al., 1997; Costa et al., 1994; Nikolaidou and Barrett, 2004; Parks and Wieschaus, 1991; Sweeton et al., 1991). Two components of the Rho pathway, *folded gastrulation* (*fog*) and *guanine nucleotide exchange factor 2* (*RhoGEF2*) exert their effects on tissue internalization by driving apical localization of myosin within invaginating cells (Dawes-Hoang et al., 2005). Salivary gland invagination defects in both *fog* and *RhoGEF2* are apparent from a delay in

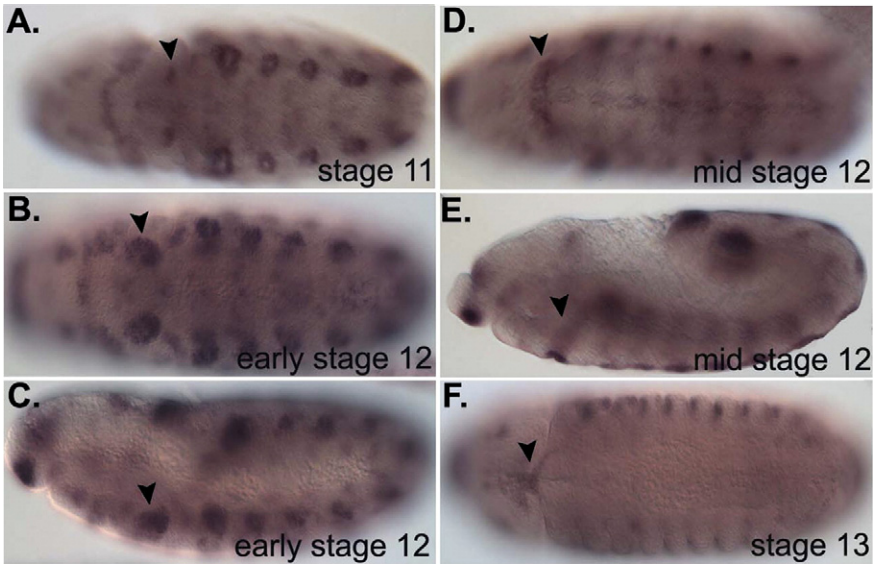

Fig. 2. *18w* RNA expression within the salivary gland. (A) Ventral view of a stage 11 embryo. *18w* RNA expression within the salivary placode begins in a small area at the dorsal-posterior edge (arrowhead). (B) Ventral and (C) the corresponding lateral view of an early stage 12 embryo. During this stage, *18w* expression spreads throughout the salivary placode (B, arrowhead) and is also evident in salivary gland cells that have invaginated (C, arrowhead). (D) Ventral and (E) the corresponding lateral view of a mid stage 12 embryo. *18w* continues to be expressed strongly in salivary gland cells that have not yet invaginated (D, arrowhead) and less intensely in the cells that have internalized (E, arrowhead). By stage 13 *18w* expression is absent from all salivary gland cells and becomes apparent within salivary duct cells (F, arrowhead).

gland internalization and the consequent anterior mislocalization of the gland as seen in *18w* mutants (compare Fig. 5B to Figs. 5C, D). Since *fog*, *RhoGEF2*, and *18w* mutant embryos exhibit similar salivary gland invagination defects, we tested for genetic interactions between *18w* and both *fog* and *RhoGEF2*. While none of the single heterozygotes display any obvious defects, embryos doubly heterozygous for *18w* and either *fog* or *RhoGEF2* exhibit salivary gland invagination defects, suggesting that *18w* might be a novel component of the Rho-mediated pathway (Table 1).

Activation of the Rho-signaling pathway results in the phosphorylation of the myosin II regulatory light chain, encoded by the *spaghetti squash* (*sqh*) gene (Karess et al., 1991; Winter et al., 2001). The phosphorylated form of Sqh can interact with actin and cause actomyosin-based contractility at the apices of cells. To verify that 18W is part of the Rho pathway, we checked whether overexpression of 18W results in an upregulation of Rho signaling, evident as an increase in phosphorylation of Sqh. Immunoblot analysis on embryonic extracts using anti-phospho-Sqh antibody reveals that overexpressing 18W throughout the embryo results in a two-fold increase of P-Sqh (Fig. 5G, lane 2) when compared to wild type (Fig. 5G, lane 3). Similar results are seen when a constitutively active form of Rho is overexpressed ubiquitously in the embryo (Fig. 5G, lane 1). Moreover, introducing one copy of a phosphomimetic *sqh* transgene, *sqhE20E21*, into an *18w* mutant rescues the *18w* invagination defects, indicating that 18W acts upstream of Sqh phosphorylation (Table 1). Thus, both genetic and biochemical evidence indicate that 18W is a novel component of the Rho signaling pathway.

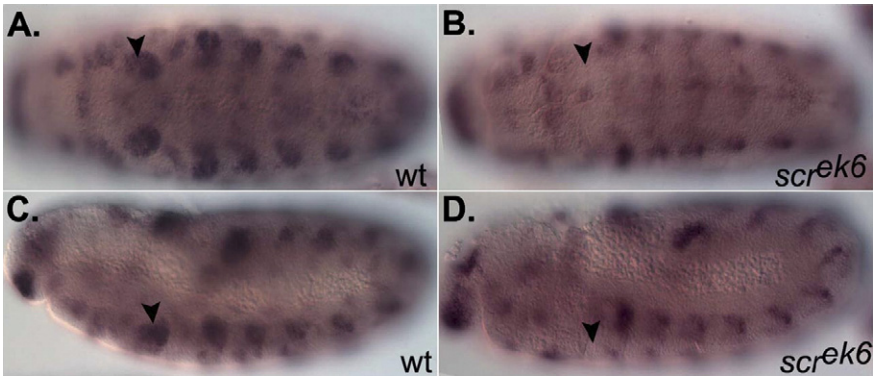

Fig. 3. *18w* expression within the salivary gland cells is dependent on *Scr*. (A, B) Ventral and (C, D) corresponding lateral views of wild-type and *Scr* mutant embryos stained against *18w* RNA. Site of salivary placode is indicated by an arrowhead. *18w* is expressed within the salivary placode in wild-type embryos (A, C) and is absent in *Scr* mutant embryos (B, D).

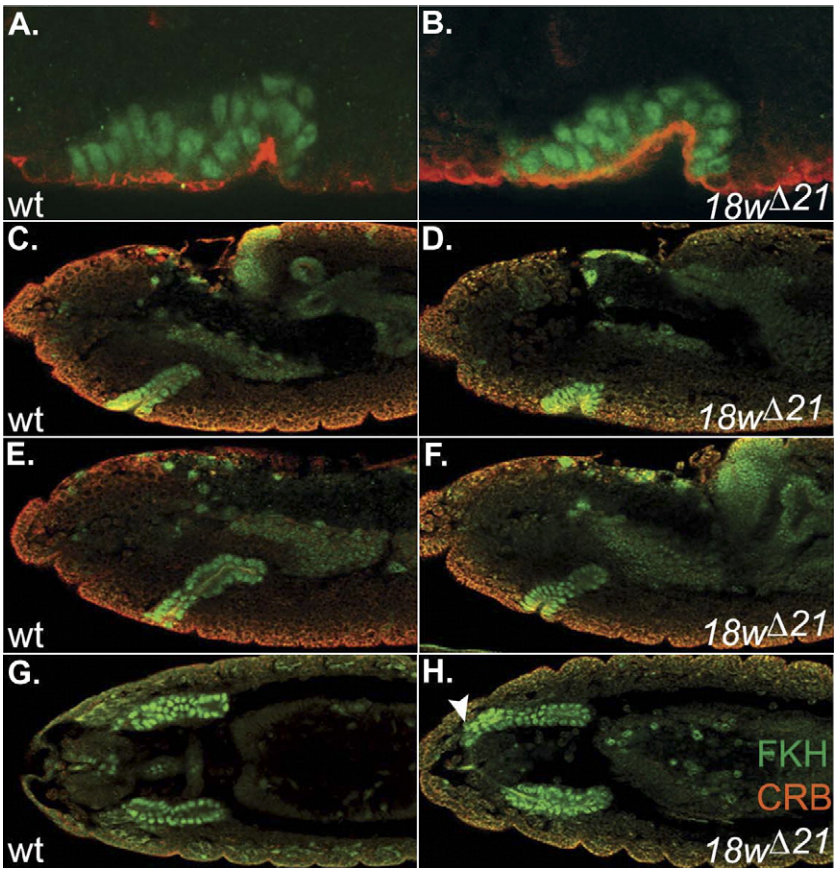

Fig. 4. *18w* mutant phenotype. Salivary gland cells are visualized with a nuclear marker FKH, in green, and the apical marker CRB, in red. In *18w* mutants, salivary gland cell internalization is uncoordinated, resulting in a much wider invagination pit (B) compared to wild type (A). Due to the invagination defects, internalization of the gland is delayed throughout stage 12 (compare D, F to C, E). Delayed internalization in *18w* mutants results in gland(s) that are more anteriorly positioned within the embryo (H, arrowhead) compared to wild type (G).

18w does not regulate Rho activity through the Fog/RhoGEF2 pathway

The activity of Rho is regulated by at least two families of molecules: RhoGEFs, which promote the exchange of GDP for GTP on Rho, thus, favoring Rho activation, and Rho GTPase activating proteins (RhoGAPs), which catalyze Rho's intrinsic hydrolysis of bound GTP to GDP, thus downregulating Rho activity. Therefore, 18W might activate the Rho pathway either by activating RhoGEF2 or by inhibiting RhoGAPs. To decipher whether 18W is part of the RhoGEF2 activating pathway, we created double mutants of *18w* with either *fog* or *RhoGEF2*. We found that both *fog*, *18w* (Fig. 5D) and *RhoGEF2*, *18w* (Fig. 5E) double mutants have more severe invagination defects than do any of the single mutants (Figs. 5C, D). While all of the cells eventually internalize in each of the single mutants, some cells remain at the surface in the double mutants. Based on this analysis, 18W does not appear to belong in the Fog/RhoGEF2 pathway. Since *fog* RNA expression within the salivary placode is unaltered in *18w* mutants, *18w* also does not regulate *fog* transcription (data not shown). Furthermore, overexpressing *fog* throughout the salivary glands in an *18w* mutant partially rescues the gland invagination defects (29% vs. 66% defects, $n=35$), demonstrating that although 18W is involved in Rho regulation, it does not lie downstream of Fog. Thus, both overexpression and double mutant analysis indicate that 18W does not regulate Rho signaling through the Fog/RhoGEF2 pathway.

Table 1
Penetrance of salivary gland defects

Genotype	wt glands (%)	Wavy glands (%)	Anterior glands (%)	No. of embryos scored
Wild type (wt)	100	0	0	60
$18w^{\Delta 21}/18w^{\Delta 21}$	34	0	66	50
$rhogef^{204291}$,+/+, $18w^{\Delta 21}$	88	0	12	52
fog^{4a6}/+; $18w^{\Delta 21}$/+	48	0	52	33
$18w^{\Delta 21}/18w^{\Delta 21}$;sqhE20E21/+	74	26	0	38
$cv\text{-}c^{M62}/cv\text{-}c^{M62}$	62	38	0	32
sca-GAL4,UAS-18w	30	40	30	70
sca-GAL4,UAS-18w; $cv\text{-}c^{M62}$/+	29	25	46	80
$18w^{\Delta 21}/18w^{\Delta 21}$; $cv\text{-}c^{M62}$/+	70	13	16	37
sca-GAL4; UAS-RhoGAP5A dsRNA	83	10	7	89
sca-GAL4/+; UAS-RhoGAP5A, $cv\text{-}c^{M62}/cv\text{-}c^{M62}$	0	81	12	49

Percent of embryos with gland migration and invagination defects.

RhoGAP5A and RhoGAP88C/cv-c are important for proper salivary gland development

To determine whether 18W negatively regulates the RhoGAP branch of the Rho pathway, we first had to identify the

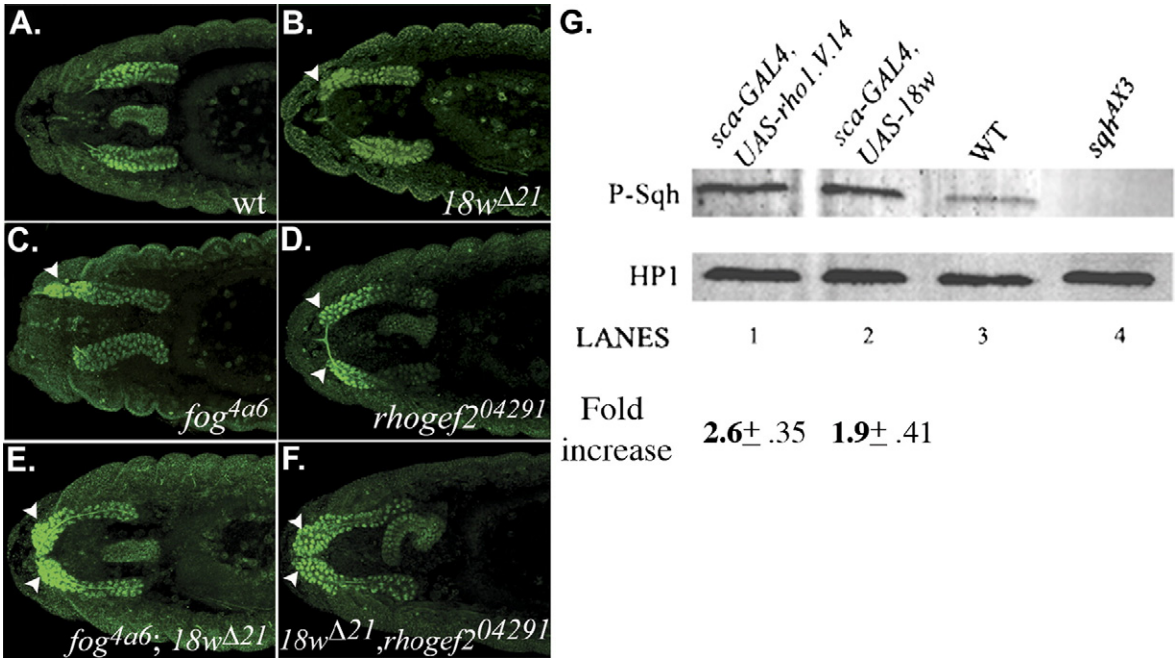

Fig. 5. *18w* is a component of the Rho signaling pathway. (A–F) Ventral views of stage 15 embryos. Salivary glands are visualized with FKH antibody in green. *fog* (C) and *rhogef2* (D) mutants have anteriorly placed gland(s) as seen in *18w* mutants (B). *fog 18w* (E) and *rhogef2 18w* (F) double mutants have more severe invagination defects compared to the single mutants. (G) Immunoblot of phosphorylated Sqh detected with anti-phospho-Sqh antibody in embryonic extracts. Lower panel is duplicate blot probed with anti-HP1 for loading control. Results from three separate experiments reveal that levels of phosphorylated Sqh are elevated in extracts that ubiquitously overexpress 18W (lane 2, 1.9±0.41-fold increase) and Rho (lane 1, 2.6±0.35-fold increase) when compared to wild-type extracts (lane 3). Phosphorylated Sqh is absent in extracts of a *sqh* null (lane 4).

RhoGAPs involved in salivary gland development. Of the 20 distinct RhoGAPs encoded by the *Drosophila* genome, we examined 17 *UAS-RhoGAP* dsRNA lines (Billuart et al., 2001). Each of these lines was crossed to flies containing a salivary gland-expressing driver, *scabrous*-GAL4, and the progeny were screened for salivary gland defects. Only RhoGAP5A dsRNA expression resulted in salivary gland defects. Invagination defects are evident from more anteriorly placed glands (Fig. 6B, Table 1), while migration defects resulted in wavy glands (Fig. 6A, Table 1). Similar defects are seen upon expressing high levels of constitutively active Rho in the salivary gland with the *scabrous*-GAL4 driver (Figs. 6C, D). Overexpressing low levels of Rho in the gland results in mostly migratory defects (data not shown), indicating that gland migration is more sensitive to the levels of Rho signaling than is the process of invagination. Invagination defects are seldom accompanied by migration defects, presumably because glands that have not properly internalized do not reach the visceral mesoderm upon which they normally migrate.

Since the GAL4/UAS dsRNA system may not result in a complete loss-of-function phenotype, we also examined strong alleles of two GAPs, RhoGAP68F and RhoGAP88C/Cv-c, which we will refer to simply as Cv-c. These have previously been shown to regulate Rho activity (Denholm et al., 2005; Sanny et al., 2006) but lack salivary defects using the dsRNA interference technique mentioned above. While neither *rhoGAP68F* nor *cv-c* mutations cause invagination defects, the *cv-c* mutants do display migration defects similar to those seen in embryos expressing RhoGAP5A dsRNA (compare Figs. 6A

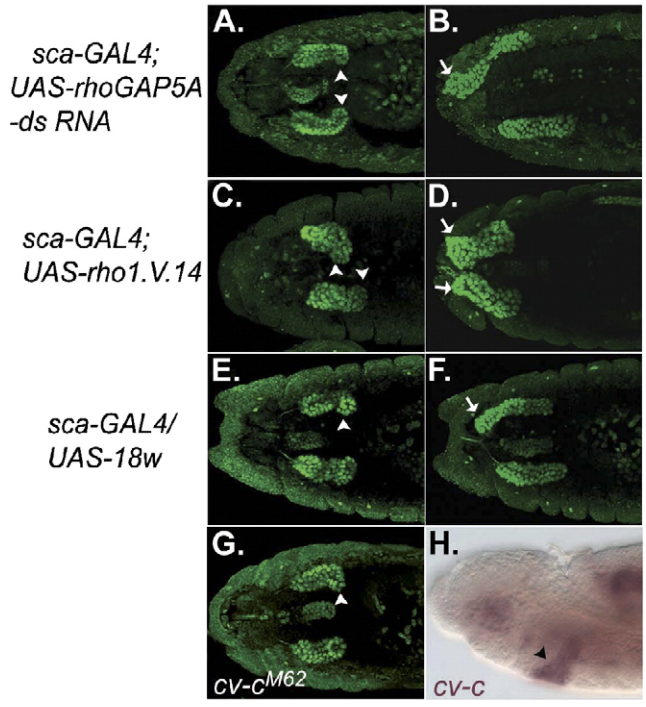

Fig. 6. Mutant phenotype of RhoGAPs-5A and -88C/Cv-c. (A–G) Ventral views of embryos stained with anti-FKH antibody in green. Expressing RhoGAP-5A dsRNA in the developing salivary gland with *scabrous-GAL4* causes either gland migration defects, which results in wavy glands (A), or invagination defects, which results in anteriorly placed glands (B). Similar defects are observed when either activated Rho (C and D) or 18W (E and F) is overexpressed in the gland with *scabrous-GAL4*. Loss of Cv-c results in migration defects (G). *cv-c* RNA *in situ* hybridization in wild-type embryos reveals that *cv-c* transcript is expressed in invaginating salivary gland cells (H).

and G, Table 1). To determine whether RhoGAP5A and Cv-c act redundantly within the salivary gland to regulate Rho activity, we expressed RhoGAP5A dsRNA within the gland in a *cv-c* mutant. These embryos should have reduced activity of both RhoGAPs. We found them to have gland invagination and migration defects that are more severe and penetrant than embryos that lack just one of them (Table 1), indicating that these RhoGAPs are, in fact, partially redundant during salivary gland development.

In situ hybridization in wild-type embryos shows that *cv-c* RNA is not maternally contributed but is expressed in several tissues undergoing morphogenesis (Denholm et al., 2005). In addition to its expression within the developing trachea and mesoderm, *cv-c*, similar to *18w*, is expressed in the salivary glands prior to and during their invagination (Fig. 6H). Unlike *18w*, however, *cv-c* expression does not appear to originate at the initial invagination site but rather initiates expression throughout most of the placode (data not shown). During the onset of invagination at stage 12, *cv-c* expression intensifies and continues to be expressed within cells that have internalized (Fig. 6H) until the conclusion of invagination at stage 13 (data not shown).

To examine whether 18W regulates Rho activity by inhibiting Cv-c, we performed several overexpression and genetic interaction experiments. As might be expected if 18W negatively regulates Cv-c, overexpressing *18w* within the salivary gland results in migratory defects similar to those seen in *cv-c* mutant embryos (Table 1). In addition to migratory defects, however, some embryos overexpressing *18w* also exhibit invagination defects, suggesting that Cv-c may not be the only RhoGAP negatively regulated by 18W. Moreover, lowering the dose of *cv-c* enhances the defects caused by *18w* overexpression and suppresses the *18w* mutant invagination defects (Table 1), further supporting the role of 18W as a negative regulator of RhoGAP signaling. Therefore, genetic interaction experiments indicate that 18W regulates Rho signaling in the salivary gland by inhibiting at least one known RhoGAP.

Discussion

Using both genetic and biochemical assays, we show that the Toll-like receptor, 18W, is required for salivary gland morphogenesis and is a novel component of the Rho-signaling pathway that leads to apical constriction. Previous studies have shown that the Fog ligand activates RhoGEF2 through an as yet unidentified receptor, leading to the apical constriction of cells that form the ventral furrow and posterior midgut (Barrett et al., 1997; Costa et al., 1994; Dawes-Hoang et al., 2005; Fig. 7). Similar to salivary gland cells in *18w* mutants, cells of the ventral furrow and posterior midgut in *fog* mutants do eventually invaginate but in an uncoordinated and delayed fashion (Costa et al., 1994). Since *18w* and *fog* mutants have similar invagination defects, and 18W is a receptor protein that activates Rho signaling, we investigated whether 18W might be the FOG receptor. This seems unlikely, however, because FOG overexpression within the salivary gland rescues the *18w* mutant salivary gland defects. Since *fog* mutations do not completely eliminate apical constriction during ventral furrow and posterior midgut formation but *RhoGEF2* mutations do, it has been argued that additional pathways must regulate apical constrictions via RhoGEF2 (Barrett et al., 1997; Hacker and Perrimon, 1998). However, since *18w RhoGEF2* double mutants have more severe defects than either of the single mutants, 18W is not one of the additional upstream activators of RhoGEF2. Although neither present downstream of FOG nor upstream of RhoGEF2, 18W does appear to be positioned upstream of Sqh phospho-

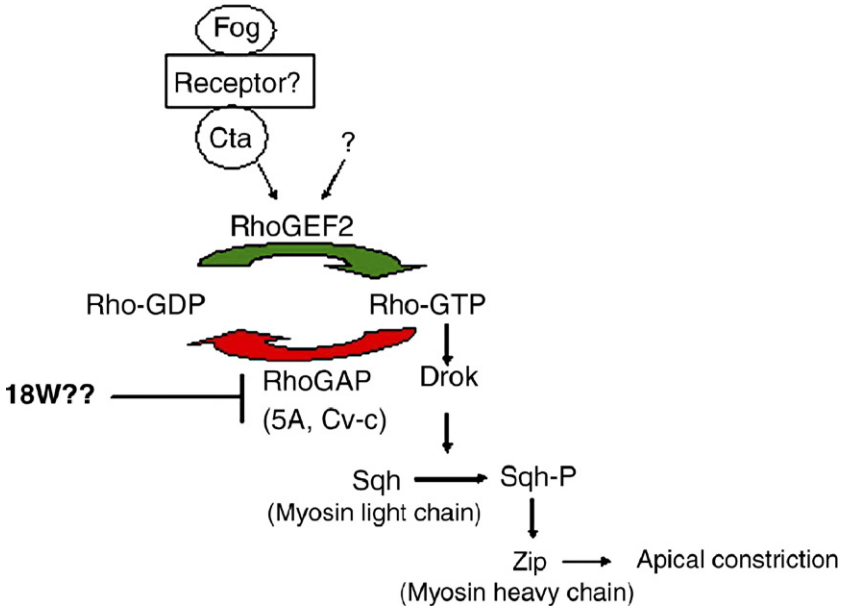

Fig. 7. Model of the Rho signaling pathway regulating apical constriction during ventral furrow, posterior midgut, and salivary gland morphogenesis. While 18W has not been linked to the Rho pathway during the development of the ventral furrow and posterior midgut, it does appear to negatively regulate RhoGAPs during salivary gland invagination.

rylation since the *18w* salivary gland mutant phenotype can be rescued by introducing one copy of a phosphomimetic allele of *sqh*.

One possible way that 18W might activate Rho signaling is by negatively regulating RhoGAPs. We identify two RhoGAPs, RhoGAP5A and Cv-c, that function partially redundantly during salivary gland morphogenesis. Embryos defective for both RhoGAPs exhibit invagination and migration defects similar to those observed when 18W is overexpressed within the salivary glands. Comparable defects are also seen upon expression of activated Rho, supporting the role of both RhoGAPs and 18W in Rho signaling.

Although overexpression and genetic interaction data demonstrate that 18W does indeed work in opposition to Cv-c activity, we have yet to decipher whether 18W actually negatively regulates RhoGAPs or if it controls Rho signaling through an alternate and unknown pathway. Another RhoGAP, RhoGAPp190, is regulated by the Src family of tyrosine kinases in both mammals and *Drosophila* (Arthur et al., 2000; Billuart et al., 2001). Depending on the site of phosphorylation, mammalian RhoGAPp190 can be either activated or inhibited by Src (Haskell et al., 2001; Roof et al., 1998), while the *Drosophila* RhoGAPp190 appears to be only negatively regulated by the *Drosophila* Src homolog, Src64B (Billuart et al., 2001). Genetic interactions and double mutant analysis with *18w* and either *Src64B* or the other *Drosophila Src* gene, *Src42A*, however, suggest 18W does not regulate RhoGAPs via Src kinases in *Drosophila* (data not shown).

Considering that 18W is a member of the Toll family of receptors, it might signal through the pathway used by Toll itself. Upon activation by its ligand, Spätzle, Toll signals via the cytoplasmic proteins MyD88, Tube, and Pelle to promote the degradation of the Cactus protein. This degradation releases the sequestered transcription factor dorsal, allowing it to enter the nucleus and activate transcription (reviewed in Morisato and Anderson, 1995). Although both *18w* and *Toll* are expressed in the salivary gland, we found no evidence to suggest that 18W signals through the Toll-pathway or that it functions redundantly with Toll. Zygotic *tube*, *pelle*, *MyD88*, or *Toll* mutant embryos do not have salivary gland defects (data not shown) and MyD88 does not physically interact with any of the Toll-like receptors except for Toll itself (Tauszig-Delamasure et al., 2002). Similarly, there are no obvious genetic interactions between mutant alleles of *18w* and *Toll* based both on lethality and salivary gland abnormalities (Eldon et al., 1994 and data not shown).

Similar to the Toll family of receptors, many RhoGAPs and RhoGEFs are found in both mammals and flies. The *Drosophila* genome encodes 21 RhoGAPs and 20 RhoGEFs but only seven Rho-family GTPases (Bernards, 2003). Since a specific Rho-GTPase can be regulated by multiple RhoGAPs, there may be some redundancy in the function of the RhoGAPs (Denholm et al., 2005; Lamarche and Hall, 1994). This appears to be the case during salivary gland development. Of the 17 RhoGAPs analyzed by RNAi, by available alleles, or by both, two resulted in distinct defects in the salivary glands. Mutant embryos that lack both of these RhoGAPs have more severe and penetrant gland defects than embryos that only lack one, indicating that the two have redundant roles during gland development.

Since 18W is expressed in several tissues undergoing morphogenesis, it will be interesting to establish whether it is important for the development of additional tissues other than the salivary gland. It will also be interesting to determine whether 18W functions in opposition to the particular RhoGAPs that are active within these other tissues. Overall, since very little is known about pathways controlling RhoGAP activity during apical constriction, identifying additional genes that interact with 18W may prove to be important not only in elucidating RhoGAP regulation but also in understanding the process of epithelial invagination.

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